APPENDIX 1

FINAL PROGRESS REPORT

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"Xenopus Microarrays: A Tool for Molecular Toxicology"

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a. Specific Aims:

Xenopus laevis is a well-established model system with ease of chemical exposure. Inter-laboratory studies demonstrated the reliability of the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (**1,2**). *Xenopus* microarray analysis holds great promise as a versatile tool for drug screening and mechanistic studies of environmental toxicology. The objective of this project was to develop *Xenopus* microarrays from ~18,500 EST clones obtained from NIEHS to suit the needs of researchers with different research objectives and laboratory facilities. In addition, utility of *X. laevis* DNA chips has been explored for *Rana pipiens*.

Aims include: (1) development of *Xenopus laevis* high density cDNA microarrays, (2) production of reference samples for array analysis, (3) exploration of the utility of the *Xenopus* cDNA microarrays using 7 classified toxicants, (4) development of diagnostic cDNA microarrays for *Xenopus* toxicology analysis, (5) development of methodology for inter-species extrapolation to the mouse, and (6) exploration of the utility of the *Xenopus laevis* cDNA microarray for *Xenopus tropicalis*.

b. Studies and Results:

Xenopus EST clones (194 96-well plates, totaling 18,624 clones) obtained from Dr. Blackshear's laboratory at NIEHS were PCR amplified and the DNA fragments were separated by agarose gel electrophoresis for evaluation of the cDNA insert size, PCR-product concentration and evidence of multiple DNA products (contamination). PCR amplification and evaluation of the cDNAs was carried out at the Genomics Technology Support Facility at Michigan State University.

Our analysis showed ~12% of the clones from these plates are contaminated. These clones were excluded from further investigation. The incidence of multiple PCR products from other clones was low (<4%). DNA concentrations from the PCR reactions were estimated for each clone by comparison with Low Mass DNA Ladder (Invitrogen) on agarose gels stained with ethidium bromide. The majority of the clones amplified successfully yielding 2-20 ug of DNA, however approximately 6% of the clones failed to amplify. Taking into account the number that failed to amplify and those exhibiting contamination, approximately 16,000 of the clones are suitable for further analysis. One hundred high density microarray slides containing of all suitable clones (~16,000) were printed using the PCR amplified DNA (**Aim 1**).

mRNA was separated from *Xenopus* blastula embryos treated with the seven classified toxicants: (1) phorbol ester, 12-myristate 13-acetate (PMA), (2) DNA damaging agent, benzo(a)pyrene-7,8-dihydrodiol (BPD), (3) non-coplanar polychlorinated biphenyl-like phenobarbital (PB), (4) peroxisome proliferator, ciprofibrate, (5) inflammatory agent, lipopolysaccaride (LPS), (6) hypoxia inducer, phenylhydrazine, and 7) aryl hydrocarbon receptor agonist, beta-naphthoflavone (BNF). Control embryos were treated with solvents of each chemicals. Reference mRNA samples were produced by mixing mRNA preparations obtained from *Xenopus* embyos treated with 7 different chemicals and 7 controls (**Aim 2**).

Eggs from 12 frogs were harvested and pooled. The pooled eggs were fertilized and were sorted for successful cleavage to 2 cells (1.5 hr after fertilization), split into 8 groups (4 for control and 4 for treatment groups of ~1,200 eggs) and visually monitored for normal morphology. Embryos were harvested at stage 8 (5 hr after fertilization). Toxicant treatment has been investigated for its affect on gene expression in the stage 8 embryos by culturing the embryos in 100 ng/ml toxicant from 2 hr after fertilization until harvest (3 hr toxicant treatment) (**Aim 3**).

Four microarray analyses were carried out for each chemical treatment group with reference design: (a) Cy5-conjugated treated embryos vs. Cy3-conjugated reference, (b) Cy3-conjugated treated embryos vs. Cy5-conjugated reference, (c) Cy5-conjugated control embryos vs. Cy3-conjugated reference and (d) Cy3-conjugated control embryos vs. Cy5-conjugated reference. In total, 28 microarray analyses were carried out (**Aim 3**).

mRNA (0.5 μg) obtained from control or treatment group and the reference mRNA sample with total volume of 20 μl were reverse-transcribed with oligo$(dT)_{18\text{-}22}$ primer and dNTPs. Cy3- or Cy5-conjugated CTP incorporated cRNA was produced from the reverse-transcribed cDNA using a Low RNA Input linear Amp kit (Agilent). The cRNA probe was purified by a RNeasy mini spin column (Qiagen). The cRNA in TE buffer were quantitated by absorbance at 260 nm and the quality will be assessed by LabChip test kits using 2100 Bioanalyzer (Agilent Technologies). The fluorescently-labeled control or experimental cRNA probe solutions (4 μg) were mixed with reversely labeled reference probes, denatured and hybridized overnight at 50°C. Microarray analyses were carried out and imaging and quantitative analysis of the microarrays was carried out using GenePix 4000A (Axon Instruments).

Genes with their expression levels statistically significantly different ($p<0.05$) from control after treatment were selected after importing the microarray analyses results into the GeneSpring software. Among ~16,000 genes, numbers of statistically significant genes in each treatment group varied from 145 to 1000 genes (**Table 1**).

Number of genes with their expression levels increased or decreased 1.5-fold and higher after chemical treatment were shown in **Table 1**. Though 980 experimental data were obtained by addition of number of genes in 7 classes, total number of genes involved with the data were 942 because there were overlapping (intersecting) genes among treatment groups. Among the 7 classes, PMA treatment of the embryos resulted in the highest number of gene expressions changed 1.5-fold and higher in embryonic stage 8 (blastula) (**Table 1**).

The gal file with identity of each spot and location on the slide fit the GenePix quantitation software. However, the gal file failed to fit the Agilent Feature Extraction Software (Agilent

Technologies) because an image obtained by the Agilent scanner was a mirror image of the image obtained from the same slide using GenePix. We communicated with the Agilent and they developed a software which could flip over the image. Number of genes significant at $p<0.05$ with fold change of 1.5 and higher by Agilent system was 496 (**Aim 3**).

Diagnostic, low density microarray were developed as a low cost product which can be used as a diagnostic tool of unknown environmental toxicants, drugs or food additives. One hundred diagnostic chips were produced by spotting in triplicate the 948 genes selected from results obtained with high density *xenopus* microarray analyses. Lambda Q-gene nucleotides were also spotted as a landmark and positive control. The 948 genes contained genes with expression levels increased, decreased or failed to change after treatment of 7 classes of toxicants as shown in **Table 1**. We produced a database using clone identity of the 948 *Xenopus* clones. By key word search, Genbank accession number of each clone was identified and, using the Genbank accession number of each clone, *Xenopus* unigene cluster was identified (**Aim 4**).

Animal experiments are commonly used to screen toxic effects of drugs and chemicals used in the household and industry and pesticides used for farming. Differentiation of developing embryos is highly conserved among vertebrates. Our hypothesis was that a highly conserved gene in mouse and *Xenopus* was similarly regulated against toxicants in early embryonic stage. Thus, oligonucleotide *Xenopus*-mouse inter-species extrapolation chip pairs were developed by selection of ortholog sequence pairs of the two species to facilitate the inter-species extrapolation.

As described above, the *Xenopus* Unigene database for each of the 948 *xenopus* genes were obtained using a Genbank accession number. This database included information on similar sequences (identified at the protein level) in other model organisms. Thus, the protein identifiers for similar proteins were extracted from mouse genes. This list was filtered to select only those proteins having >= 40% similarity to the *Xenopus* sequence and also those having an NCBI RefSeq protein identifier. From the *Xenopus* RefSeq protein identifiers, DAVID (a program from the NIH) was used to find the associated mouse mRNA RefSeq sequence identifiers. We found redundancy in the list due to several factors including numerous cases where multiple *Xenopus* clones were associated with the same mouse gene and where several mouse mRNA sequences were associated with a single mouse gene due to splice variations (**Aim 5**).

A non-redundant list was produced for 194 mouse mRNA sequences with matching *Xenopus* sequences (**Table 2**). The table also revealed names or functions of the mouse genes. Names or functions of the 194 *Xenopus* genes are shown in **Table 3**. By pairing the *Xenopus* sequences with sequences of mouse or other species in the Unigene database, putative functions of *Xenopus* genes could also be deduced.

Using the mRNA sequences, 60-mer oligonucleotides for both mouse and *Xenopus* were designed for interspecies oligo microarray slide production. Oligonucleotide sequences selected from the 194 *Xenopus* and mouse genes are shown in **Tables 3 and 4**, respectively. Melting points (Tm) of the oligonucleotides were set to be $\pm$2.5°C from the median Tm value.

Total RNA samples from frog embryos after phenobarbital treatment at blastula stage were obtained as described above. Mouse (CD-1) embryos were obtained from over-ovulated mice (Charles River Labs.) and treated with phenobarbital (100 ng/ml) at blastula stage (~90-100 hr after fertilization) to be used for interspecies microarray analyses (**Aim 5**). Previous attempts by other researchers to produce a single chip for use of both species failed because a perfectly conserved stretch of 60-mer unique to a gene in both species was rare. Thus, they had to use ortholog sequences which were conserved but not with 100% sequence identity for their inter-species extrapolation chip pairs.

Initial probing across species to evaluate application of the *Xenopus* microarray to other species was investigated in collaboration with Dr. Ken Storey (Carleton University, Canada). Hepatic

RNA obtained from the North American frog *Rana pipiens* was used to probe the *Xenopus* microarray. Substantial signal was selectively retained on the *Xenopus* microarray with the probe made from *Rana pipiens* liver RNA. In addition to the frog *Rana pipiens, liver tissue of X. tropicalis*, closely related to *X. laevis,* has been obtained for a microarray analysis (**Aim 6**).

c. **Publications:** A patent entitled "Detection and Identification of Toxicants by Measurement of Gene Expression Profile" was submitted and is pending (see an attached published patent application).

d. **Project-Generated Resources:** About 16,000 unique *Xenopus laevis* PCR-replicated cDNAs, ~60 sequence-verified *Xenopus laevis* clones, high density and diagnostic microarray analyses, databases for *Xenopus* genes and *Xenopus*-mouse pairs susceptible to chemical insults, *Xenopus* reference RNA.

e. **Human subjects:** Not applicable

Literature Cited

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2. Fort, J.F., Stover, E.L., Bantle, J.A., and Finch, R.A. Evaluation of the developmental toxicity of thalidomite using frog embryo teratogenesis assay- Xenopus (FETAX): biotransformation and detoxification. Teratogen., Carcinogen. Mutagen. 20: 35-47 (2000).

Table 1. mRNA levels changed after treatment of *xenopus* embryos with 7 classes of toxicants.

Class	Toxicant	No. genes significant at $p<0.05$	No. genes significant at $p<0.05$ with fold change of 1.5 and higher
I	PMA	583	291
II	BPD	221	71
III	PB	1000	167
IV	Ciprofibrate	312	105
V	LPS	364	118
VI	Phenylhydrazine	512	199
VII	BNF	145	29